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THE REGULATION OF A POST-TRANSLATIONAL PEPTIDE ACETYLTRANSFERASE: STRATEGIES FOR SELECTIVELY MODIFYING THE BIOLOGICAL ACTIVITY OF NEURAL AND ENDOCRINE PEPTIDES

FINAL REPORT

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The broad objective of this research is to develop new strategies for pharmacologically modifying synaptic transmission by pepti-dergic neurons. It is based on the principal that post-trans-lational processing determines the biological activity of neural and endocrine peptides and uses the ß-endorphin processing pathway as a model for study. Five primary objectives were met during the course of this research. First, we found that post-translational processing enzymes are selectively and individually regulated, which indicates that certain processing enzymes serve a rate limiting role in peptide biosynthesis, while others do not. Second, pharmacologic agents targeted on cell surface receptors selectively regulate ß-endorphin processing enzymes, producing distinct changes in the molecular forms and, hence, the biological activities of the ß-endorphin peptides released from the pituitary gland. Third, brain ß-endorphin processing is also regulated through receptor activation although, in general, it appears to be more resistant to this strategy, emphasizing the need to develop agents which control peptide processing through direct enzyme inhibition or activation. Fourth, we characterized the ß-endorphin processing

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pathway in human brain and found, that despite critical differences in primary sequence, β-endorphin is processed similarly in both rat and human, documenting the relevance of studies in rats to human neurobiology. Fifth, we examined the functional consequences of β-endorphin processing and found that C-terminal shortening of β-endorphin-1-31 to β-endorphin-1-27 potentiates its central hypotensive potency. This contrasts studies on analgesia where β-endorphin-1-27 is a weak agonist, but a potent antagonist, and indicates that peptide processing can produce entirely different changes in bioactivity depending upon the postsynaptic receptors which mediate the response. Together, these findings emphasize the critical importance of processing enzymes in regulating peptide bioactivity and establish the feasibility of utilizing pharmacologic agents targeted on these enzymes to control the function of peptidergic neurons and endocrine cells.

FOREWORD

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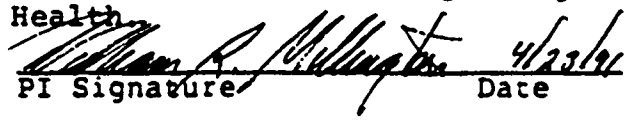

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INTRODUCTION

The discovery that biological peptides act as synaptic neurotransmitters initially offered considerable promise for the development of a whole new generation of centrally active drugs. But the prospect of using peptides as templates for drugs has born little fruit, primarily because peptides do not permeate the blood-brain barrier or other biological membranes and because they are rapidly metabolized. These limitations prompted us to consider whether a closer evaluation of the pre-synaptic mechanisms which govern peptide biosynthesis and post-translational processing might lead to alternative approaches for designing therapeutic agents to modify synaptic transmission by peptidergic neurons. The broad objective of this research program is, therefore, to create the data base necessary to develop new strategies for pharmacologically modifying the function of peptidergic neurons and endocrine cells.

Neuropeptides are initially synthesized as large, biologically inactive precursors which must undergo enzymatic processing to yield their biologically active peptide products. Studying the enzymatic processing of peptide precursors can be exceedingly complex, however; for example, pro-opiomelanocortin (POMC), which for many years has served as a prototype for studying peptide biosynthesis, is enzymatically processed to over twenty different peptides (O'Donohue and Dorsa, 1982; Millington and Chronwall, 1989). For this reason, we focused our research on the post-translational processing of β-endorphin, a product of POMC.

A. The Post-Translational Processing of β-Endorphin:

β-endorphin is initially synthesized as a thirty-one amino acid peptide, which is then further processed through N-terminal acetylation sequential C-terminal proteolysis to form up to six structural analogs; N-acetylated and des-acetyl β-endorphin-1-31, β-endorphin-1-27 and β-endorphin-1-26 (Fig. 1) (Zakarian and Smyth, 1979; Eipper and Mains, 1981; Millington et al., 1987). The sequential processing of β-endorphin is heterogeneous, which means that all of these molecular forms co-exist, in varying proportions, in the brain, pituitary and certain peripheral tissues (Zakarian and Smyth, 1982; Dennis et al., 1983).

Three enzymes are required to post-translationally process β-endorphin; a peptide specific N-acetyltransferase which N-acetylates β-endorphin peptides, an endopeptidase which converts β-endorphin-1-31 to β-endorphin-1-27 and a carboxypeptidase which removes the C-terminal histidine residue from β-endorphin-1-27, forming β-endorphin-1-26 (Eipper and Mains, 1981). When we initiated these studies, only one of these enzymes, peptide acetyltransferase, had been characterized; neither the endopeptidase nor the carboxypeptidase enzymes had been conclusively identified although recent evidence suggests that carboxypeptidase H, the enzyme which removes C-terminal lysine and arginine residues

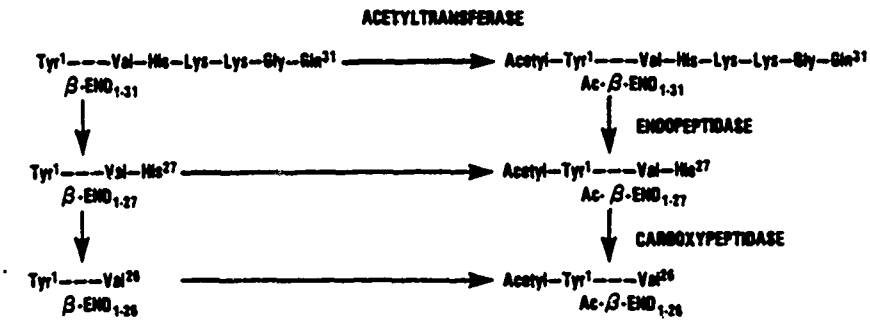

Figure 1. The Post-Translational Processing of β-Endorphin

following the endoproteolytic cleavage of peptide prohormones, also converts β-endorphin-1-27 to β-endorphin-1-26 (Smyth et al., 1989). Consequently, our research focused on peptide acetyltransferase as a 'model' for studying the regulation of post-translational processing enzymes.

The N-acetylation of β-endorphin by peptide acetyltransferase is an intriguing post-translational modification because it essentially abolishes the peptide's opioid potency. C-terminal proteolysis also eliminates opioid activity; moreover, it converts the peptide into an opioid receptor antagonist, β-endorphin-1-27, which is 4-5 times more potent than the opiate antagonist, naloxone (Nicolas and Li, 1985). β-endorphin-1-26, on the other hand, is devoid of both agonist and antagonist activities. Thus, the β-endorphin processing pathway clearly illustrates the principle that enzymatic processing determines biological activity; even relatively minor changes in structure produce profound changes in bioactivity.

B. Objectives:

The principal objective of this research was to establish the feasibility of modifying the function of peptidergic systems by using chemical agents to induce selective changes in the activity of the enzymes which post-translationally process neural and endocrine peptides. When we initiated this research, however, little was known about the regulation of processing enzymes or the role of enzyme regulation in the overall control of peptide biosynthesis. We, therefore, began by asking the basic question, 'are post-translational processing enzymes regulated?' This is a fundamental question because closely regulated enzymes are the rate limiting steps in biosynthetic pathways and commonly provide effective target sites for drug action. Thus, to establish the feasibility of pharmacologically controlling processing enzyme activity, either through cell surface receptor activation or by means of direct enzyme inhibitors, it was first necessary to determine whether processing enzymes serve a rate limiting role in peptide synthesis.

What are the criteria for establishing that an enzyme is rate limiting in a biochemical pathway? Consider catecholamine biosynthesis, for example. It is well understood that tyrosine hydroxylase (TOH) is the rate limiting step in dopamine synthesis, rather than the second enzyme in the pathway, L-aromatic amino acid decarboxylase (LAAD). This designation satisfies three criteria. First, kinetic studies demonstrated that TOH activity (Vmax) is the lowest in the pathway, approximately two orders of magnitude lower than LAAD (Thoenen et al., 1971). Accordingly, TOH inhibitors effectively block dopamine synthesis in vivo, while LAAD inhibitors are much less effective. Second, TOH activity is inducible, that is, long-term changes in dopamine synthesis increase the enzyme Vmax. LAAD, on the other hand, is not inducible. Third, the rate of tyrosine hydroxylation is not affected by substrate availability, this is an enzyme regulatory step, but the rate of DOPA decarboxylation is entirely substrate driven. Thus, the concentration of DOPA, the substrate for LAAD, is very low; it is entirely converted to product as fast as it is synthesized by TOH.

We decided to use these same criteria to establish whether peptide acetyltransferase and other β-endorphin processing enzymes served a rate limiting role in the synthesis of β-endorphin peptides. Criterion #3 had already been met; relatively high concentrations of peptide acetyltransferase substrates, des-acetyl-β-endorphin peptides, are present in the brain and intermediate pituitary, suggesting that the enzymatic reaction was not substrate driven but, like tyrosine hydroxylase, peptide acetyltransferase may serve a rate limiting function. Two criteria remained; to establish the enzyme's kinetic parameters (Vmax, Km), relative to other enzymes in the β-endorphin processing pathway, and to test whether peptide acetyltransferase activity could be induced by treatments which accelerate POMC biosynthesis.

The β-endorphin neural and endocrine system is an ideal model for studying addressing these questions because a large data base is already available from previous investigations and because the availability of a relatively homogeneous tissue, the intermediate pituitary, that synthesizes high levels of β-endorphin and its processing enzymes, facilitates experimentation. β-Endorphin's well-defined role in analgesia, cardiovascular regulation and other physiological functions also facilitates studies on the role of post-translationally in defining the biological actions β-endorphin peptides. The results of this, as well as previous studies, indicate that the development of pharmacologic agents targeted on β-endorphin processing enzymes may, in the longer term, be of distinct clinical utility.

Accordingly, after establishing how peptide acetyltransferase is regulated in the intermediate lobe, we formulated several additional objectives. First, to test whether brain β-endorphin processing is similarly regulated; second, to establish the applicability of our data to human neurobiology by evaluating the β-endorphin processing pattern in human brain; and third, to

further examine the functional consequences of N-acetylation and other β-endorphin processing steps by evaluating the central cardioregulatory actions of β-endorphin peptides.

C. Overview of the Principal Research Findings:

We initiated studies of β-endorphin processing enzyme by using the intermediate pituitary to study the regulation of peptide acetyltransferase, the enzyme which N-acetylates β-endorphin. The intermediate lobe is an ideal model system. A homogeneous tissue, controlled primarily by inhibitory dopaminergic neurons, it processes β-endorphin in a pattern similar to that of brain (Millington and Chronwall, 1989). We found that peptide acetyltransferase is indeed regulated; chronic treatment with dopaminergic drugs produced coordinated changes in peptide acetyltransferase activity, POMC gene expression and β-endorphin secretion (Millington et al., 1986). We also found, however, that carboxypeptidase H, the enzyme thought to convert β-endorphin-1-27 to 1-26, is not similarly regulated. Moreover, the differential regulation of these two enzymes induced selective changes in the molecular forms of β-endorphin released from the intermediate lobe (Millington et al., 1987). These experiments clearly demonstrated that peptide processing enzymes are individually regulated and established the feasibility of using pharmacological agents acting at synaptic receptors to selectively control the processing, and hence the biological activity, of β-endorphin peptides.

These studies also revealed that the regulation of β-endorphin processing is but one component of a series of temporally related intra- and intercellular mechanisms used by the intermediate lobe to modify POMC biosynthesis. We combined several coordinated methodological approaches to investigate intermediate lobe function, including dot blot and in situ hybridization, light and electron microscopic morphometric analysis, ^{3}H-thymidine uptake measurement and radioimmunoassay (Chronwall et al., 1987 & 1988; Millington and Chronwall, 1989). This revealed that acute treatment with dopamine receptor antagonists accelerates POMC gene transcription, elevating POMC mRNA levels, and increasing both the synthesis and secretion of β-endorphin. Subchronic treatment continues to accelerate POMC biosynthesis and also converts biosynthetically quiescent 'light' cells into 'dark' cells, those actively engaged in synthesizing POMC, thus further expanding the biosynthetic capacity of the gland as a whole. Following chronic treatment, the intermediate lobe exhibits cellular hyperplasia, an increase in the number of cells in the gland. Finally, after extended periods of interrupted dopaminergic innervation, the rate of POMC synthesis within individual intermediate lobe cells returns to control levels and cellular hyperplasia alone maintains elevated β-endorphin output from the gland (Millington et al., 1988; Dybdal et al., 1988). Thus, the intermediate lobe utilizes several independent, yet coordinated, mechanisms to modulate its total output of peptide hormones. Interestingly, alterations in β-endorphin processing occur only during chronic conditions. The selective regulation of β-endorphin

processing apparently provides a mechanism for encoding information about the temporal nature of environmental stimuli, specifically, certain stressful conditions (Berkenbosch et al., 1984), perceived by the central nervous system and transmitted to peripheral tissues, including the immune system, by the intermediate pituitary.

Upon completing investigations of the endocrine β-endorphin system, we initiated studies of β-endorphin processing in brain. Our first objective was to characterize the distribution and regulation of β-endorphin peptides. Briefly, we found that all the β-endorphin forms identified in the pituitary are present in brain, although their relative proportions vary among brain regions. This indicates that peptide acetyltransferase and other β-endorphin processing enzymes are expressed in brain and that their regulation is regionally specific. Furthermore, the enzymes apparently can be controlled in brain, as in the pituitary, by synaptic activation, based on the finding that dopaminergic drugs also modify brain β-endorphin processing; however, other pharmacologic treatments thought to produce changes in POMC biosynthesis, including chronic morphine or estradiol administration, were ineffective. Neuronal β-endorphin processing also changes physiologically, during both early maturation (Martensz, 1985) and the aging process (Wilkinson and Dorsa, 1986) although the specific modifications differ from those occurring in the pituitary (Alessi et al., 1983; Martensz, 1985). Together, these findings suggest that the regulation of neuronal β-endorphin processing differs from that of the pituitary (Berglund et al., 1989).

Although these findings suggest that pharmacologic agents targeted on β-endorphin processing enzymes may have potential clinical utility, this relies on the assumption that the β-endorphin processing pathway is the same in human brain as that of laboratory animals. Critical species differences in the primary structure of β-endorphin suggests that this may not be the case, however (Li, 1984). Specifically, substitution of the C-terminal histidine residue of β-endorphin-1-27 in the rat by tyrosine in human β-endorphin-1-27 has led to the assumption that β-endorphin-1-27 is not converted to β-endorphin-1-26 in the human, based on evidence that β-endorphin-1-26 is absent from the human pituitary. We found, however, that all six β-endorphin forms, including β-endorphin-1-26, are present in the human. Thus, despite important differences in primary sequence, β-endorphin is processed similarly in both human and rat brain.

The observation that β-endorphin processing can be altered both physiologically and pharmacologically prompted us to study the functional consequences of these regulatory processes. The role of processing in mediating the analgetic activity of β-endorphin is well understood (Nicolas and Li, 1985); however β-endorphin neurons are not solely involved in analgesia, but mediate other physiological and behavioral responses as well (O'Donohue and Dorsa, 1982). To address this question, we examined the central cardiovascular effects of β-endorphin peptides (Hirsch and Millington, 1990). We found, as others had shown previously, that

like morphine, β-endorphin-1-31 lowered mean arterial pressure (MAP) when injected intracisternally. Unexpectedly, β-endorphin-1-27 had the same effect; moreover, it was nearly 10-fold more potent than β-endorphin-1-31. As in the case of analgesia, both N-acetylation and further C-terminal proteolysis to β-endorphin-1-26 abolished the cardioregulatory activity of the peptide. This structure function relationship is quite similar to that of non-opioid β-endorphin binding sites first identified in peripheral tissues (Schulz et al., 1981; Huidobro-Toro, et al., 1982). Thus, the response to neuronally released β-endorphin peptides apparently depends on both the peptide forms produced through pre-synaptic processing and the nature of the receptors which mediate the post-synaptic response.

RESULTS AND DISCUSSION

A. The Regulation of Peptide Acetyltransferase:

Millington, W.R., O'Donohue, T.L., Chappell, M.C., Roberts, J.L. and Mueller, G.P. Coordinate regulation of peptide acetyl-transferase activity and proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary. Endocrinology 118: 2024-2033, 1986.

1. Long-term regulation: The objective of these studies was to determine whether β-endorphin processing enzymes, specifically, peptide acetyltransferase, undergoes classical enzyme induction; that is, whether treatments which accelerate the rate of POMC biosynthesis also produce parallel changes in peptide acetyl-transferase. The basic, underlying question was whether peptide acetyltransferase serves a rate limiting role in peptide biosynthesis; one of the first criteria in addressing this question is whether the enzyme in question is regulated. At the time that we initiated these studies, however, there was little experimental basis for predicting the results. We postulated three potential outcomes. First, that peptide acetyltransferase is not rate limiting and, therefore, not inducible; second, that the enzyme is rate limiting, but not regulated, such that changes in POMC biosynthesis would produce parallel changes in the ratio of acetylated to non-acetylated β-endorphin peptides; third, that peptide acetyl-transferase is both rate limiting and regulated such that changes in biosynthetic activity will maintain the extent of β-endorphin acetylation. This latter hypothesis proved to be correct.

To test the hypothesis that peptide acetyltransferase undergoes long-term regulation, we used the intermediate lobe of the pituitary gland. Earlier studies in our laboratory had shown that acute treatment with the dopamine receptor antagonist, haloperidol, stimulates intermediate lobe β-endorphin release by interrupting the inhibitory dopaminergic control of the gland (Farah, et al., 1982); chronic treatment produces a sustained increase in

secretion, inducing a compensatory increase in the rate of POMC biosynthesis and elevating the content of POMC-derived peptides (Hollt and Bergmann, 1982; Chen et al., 1983).

We found that peptide acetyltransferase does, indeed, undergo long-term enzyme induction; chronic haloperidol treatment produced a significant increase in intermediate lobe peptide acetyltransferase activity which was parallel and essentially equivalent to elevations in β-endorphin and POMC mRNA levels in the gland (Fig. 2). Time-course and dose-response studies with haloperidol further demonstrated that peptide acetyltransferase activity co-varied with POMC mRNA and peptide levels in the intermediate lobe. Chronic treatment with bromocriptine, a dopamine receptor agonist, had the opposite effects; it lowered peptide acetyltransferase activity, POMC mRNA levels and α-MSH and β-endorphin immunoreactivity (Fig. 2). Kinetic studies further showed that haloperidol treatment increased the Vmax of peptide acetyltransferase without affecting the Km of the enzyme for either of its substrates, des-acetyl-α-MSH or acetyl coenzyme A (Table 1). This suggests that haloperidol treatment induced peptide acetyltransferase activity by increasing the amount of the enzyme present in the intermediate lobe, rather than by altering the substrate affinity of existing enzyme molecules. Subcellular fractionation studies also demonstrated that haloperidol elevated the specific activity of secretory vesicle associated peptide acetyltransferase without affecting the specific activities of acetyltransferases unrelated to post-translational peptide processing present in the endoplasmic reticulum and cytosol. These results suggest that peptide acetyltransferase activity and POMC biosynthesis are co-regulated.

Having established that peptide acetyltransferase undergoes enzyme induction, we next considered whether other post-translational processing enzymes are similarly regulated. To test this, we examined the regulation of carboxypeptidase H (in collaboration with Dr. Vivian Hook, USUHS), the enzyme which removes C-terminal arginine and lysine residues after endoproteolytic cleavage of POMC and other prohormones. Importantly, carboxypeptidase H is also thought to catalyze removal of the C-terminal histidine residue from β-endorphin-1-27, forming β-endorphin-1-26 (Smyth et al., 1989). We found that, unlike peptide acetyltransferase, carboxypeptidase H is not induced by chronic haloperidol treatment (Fig. 3). That peptide acetyltransferase, but not carboxypeptidase H, is induced by dopaminergic antagonists suggests that POMC processing enzymes are selectively and individually regulated.

2. Short-term regulation: Subsequently, we tested whether peptide acetyltransferase activity undergoes short term regulation by chemical agents which stimulate intermediate lobe β-endorphin secretion. Short term activation, mediated by alterations in Km, but not in Vmax, has been demonstrated for carboxypeptidase H (Hook et al., 1985) but whether this mechanism regulates other post-translational processing enzymes is unknown. Our initial, in vivo studies indicated that acute haloperidol treatment had no effect

on peptide acetyltransferase; however, measuring short term changes in enzyme activity can be problematic because they may be mediated through changes in ionic or second messenger concentrations which are lost when the internal milieu of intermediate lobe secretory vesicles is disrupted. We, therefore, measured the precursors and products of peptide acetyltransferase N-acetylated and des-acetyl-β-endorphin peptides using two specific radioimmunoassays, one which measures total β-endorphin immunoreactivity and a second which detects only N-acetylated β-endorphin peptides.

We examined the short-term regulation of peptide acetyltransferase using three separate experimental paradigms, selecting time points at which POMC biosynthesis is unaffected. First, for in vivo studies, rats were treated with a single acute injection of haloperidol, (0.1 - 10.0 mg/kg), isoproterenol (10 - 300 μg/kg) or vehicle and were killed one hour later. Second, neurointermediate lobes (NIL) were incubated in vitro with either isoproterenol (1.0 μM), which stimulates secretion, or dopamine (50 μM), which inhibits it, for up to five hours and β-endorphin peptides were measured at 20 min intervals in the incubation medium and, at the end of the experiment, in the NIL. The third experiment used a similar protocol except that primary intermediate lobe cultures were used. In all three experiments, however, the ratio of N-acetylated to total β-endorphin peptides was not changed by any of the drug treatments in either the NIL or the release media. Thus, while carboxypeptidase H is regulated by short, but not by long-term induction, peptide acetyltransferase, conversely, clearly undergoes long term induction but does not appear to be regulated by short term changes in the secretory activity of the intermediate pituitary.

B. Dopaminergic Agents Selectively Alter β-Edorphin Processing:

Millington, W.R., O'Donohue, T.L. and Mueller, G.P. Dopaminergic agents selectively alter the post-translational processing of β-endorphin in the intermediate pituitary of the rat. J. Pharmacol. Exp. Ther. 243:160-170, 1987.

Ultimately, to interpret the significance of processing enzyme regulation, it is necessary to examine the effects it produces on the products of the enzymatic reaction. To examine this question, we tested whether chronic treatment with dopaminergic agents alters the molecular forms of β-endorphin in the intermediate lobe. Based on our initial working hypothesis, and on the finding that peptide acetyltransferase is coordinately regulated with POMC synthesis, we predicted that dopaminergic agents would produce parallel changes in β-endorphin levels and acetylation, leaving unchanged the ratio of acetylated to non-acetylated β-endorphin forms. This turned out to be the case.

We first examined the acetylation of β-endorphin using radioimmunoassays specific for total and N-acetylated β-endorphin peptides as described earlier. Next, we separated the individual

molecular forms of β-endorphin using cation exchange chromatography. Both experiments showed that neither haloperidol nor bromocriptine treatments had any effect on the extent to which β-endorphin is N-acetylated; N-acetyl-β-endorphin peptides constituted approximately ninety percent of total β-endorphin immunoreactivity under both control and treated conditions. These results support the hypothesis that the coordinate regulation of peptide acetyltransferase activity and β-endorphin biosynthesis functions as a mechanism for maintaining the acetylation of β-endorphin peptides independent of changes in the secretory activity of the intermediate pituitary.

Although dopaminergic agents did not alter β-endorphin acetylation, they did produce distinct changes in the relative concentrations of individual β-endorphin peptides in the NIL. Cation exchange chromatography revealed that chronic haloperidol treatment substantially elevated the concentrations of β-endorphin-1-31, N-acetyl-β-endorphin-1-31 and N-acetyl-β-endorphin-1-27, but in contrast, N-acetyl-β-endorphin-1-26 was not significantly affected (Fig. 4). Bromocriptine treatment had the opposite effect; it depleted β-endorphin-1-31, N-acetyl-β-endorphin-1-31 and N-acetyl-β-endorphin-1-27 but not N-acetyl-β-endorphin-1-26 concentrations (Fig. 4). β-Endorphin peptides were secreted from the NIL in vitro in the same relative proportion as they are contained in the gland, both in controls and following chronic haloperidol, indicating that dopaminergic induced alterations in β-endorphin processing result from specific changes in processing and not from the preferential release of one or more molecular forms of the peptide. Examination of the ratio of the major molecular forms of β-endorphin in the NIL (the ratio of β-endorphin-1-31 : N-acetyl-β-endorphin-1-31 : N-acetyl-β-endorphin-1-27 : N-acetyl-β-endorphin-1-26 was 1 : 2.5 : 6.8 : 6.1 in control rats and 1 : 2.8 : 6.8 : 3.1 following haloperidol) indicates that haloperidol selectively alters the relative amount of N-acetyl-β-endorphin-1-26 in the NIL, producing little or not change in the proportions of the other peptides. These data indicate that the conversion of N-acetyl-β-endorphin-1-27 to N-acetyl-β-endorphin-1-26 is selectively regulated by dopaminergic receptor activity. This finding is consistent with the observation the carboxypeptidase H, the enzyme which catalyzes the conversion of β-endorphin-1-27 to β-endorphin-1-26 (Smyth et al., 1989) is not induced by haloperidol and thus limits the formation of N-acetyl-β-endorphin-1-26. Together, these data support the hypothesis that the specific ratio of β-endorphin peptides is determined by the selective regulation of β-endorphin processing enzymes.

We also examined the effect of dopaminergic agents on two additional β-endorphin-derived peptides, α-endorphin (β-endorphin-1-16) and gamma-endorphin (β-endorphin-1-17). We found that the concentrations of these peptides were also elevated by D-2 dopamine receptor antagonists, and reduced by D-2 agonists, in the NIL. Similar to its effects on β-endorphin peptides, haloperidol did not alter the extent of acetylation of α-endorphin and gamma-endorphin. However, acute haloperidol administration, which had no differen-

tial effect on β-endorphin peptides, led to the selective depletion of gamma-endorphin in the NIL. These data are consistent with reports that α- and gamma-endorphin synthesis is a late post-translational event; the peptides are therefore localized specifically in mature secretory vesicles, localized near the periphery of intermediate lobe melanotrophs, which are preferentially released. Interestingly, these smaller forms of β-endorphin exhibit a behavioral activity spectrum comparable to that of classical psychostimulants, such as amphetamine (de Wied and Jolles, 1982)

C. Coordinate Regulation of Peptide Acetyltransferase Activity and POMC Biosynthesis:

1. Dopaminergic regulation of the biosynthetic activity of individual melanotrophs.

Chronwall, B.M., Millington, W.R., Griffin, W.S.T., Unnerstall, J. and O'Donohue, T.L. Histological evaluation of the dopaminergic regulation of proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary involving in situ hybridization and 3H-thymidine uptake measurement. Endocrinology 120:1201-1211, 1987.

Chronwall, B.M., Hook, G.R. and Millington, W.R. Dopaminergic regulation of the biosynthetic activity of individual melanotropes in the rat pituitary intermediate lobe: A morphometric analysis by light and electron microscopy and in situ hybridization. Endocrinology 123:1992-2002, 1988.

The initial objective of these studies was to evaluate haloperidol and bromocriptine induced changes in POMC gene expression using in situ hybridization, a technique in which POMC mRNA is detected by hybridizing a cDNA probe directly on tissue sections, thereby quantifying changes in POMC mRNA within individual cells of the intermediate pituitary. This would enable us to investigate whether coordinate changes in POMC gene expression and peptide acetyltransferase activity were cell specific or if they occurred equally in all intermediate lobe cells. The results confirm our earlier experiments with dot-blot hybridization assays which demonstrated that long term haloperidol treatment elevates, and bromocriptine reduces, POMC mRNA levels in all cells of the intermediate lobe. However, these studies produced several additional, unexpected findings showing that dopaminergic agents induce a coordinated set of biochemical and cellular responses which are manifested according to a distinct temporal pattern following acute, subchronic and chronic treatment.

The initial results of these studies revealed, unexpectedly, that dopaminergic agents produced quite rapid changes in POMC mRNA levels. Haloperidol (2 mg/kg) elevated POMC mRNA, not only after chronic administration, as we had previously shown (Millington et al., 1986), but also after acute (6 h) and subchronic treatments

(twice daily for 2 d) demonstrated that POMC mRNA was elevated within only four hours of a single haloperidol injection (5 mg/kg) and remained above control values for twenty-four hours; after a lower dose (2 mg/kg) it was elevated only transiently. This indicates that acute blockade of dopamine receptors produces rapid changes in POMC gene transcrip-tion, perhaps within minutes of treatment.

The second finding revealed by in situ hybridization was that POMC mRNA is not uniformly distributed among intermediate lobe cells. POMC mRNA was heterogeneously distributed among individual cells of the gland, suggesting that the intermediate lobe contains two classes of cells maintaining differing rates of POMC biosynthesis. Subchronic haloperidol treatment not only increased the amount of POMC mRNA in each cell but also eliminated the heterogeneity in its cellular distribution. Bromocriptine lowered POMC mRNA levels and also reduced this cellular heterogeneity. This further indicates that the heterogeneous distribution of POMC mRNA was not an artifact resulting from differences in tissue thickness or other experimental variables.

To further test the concept that intermediate lobe cells synthesize POMC at different rates, we conducted morphometric studies using both light and electron microscopy. Both approaches showed that intermediate lobe melanotrophs differ in the tinctorial properties of their cytoplasm; some cells appeared distinctly darker, others lighter. Morphometric studies showed why this was so. Darkly staining cells have a denser cytosol and contain a significantly greater amount of subcellular organelles, including rough endoplasmic reticulum, Golgi complex, secretory vesicles and mitochondria, involved in synthesizing and storing secretory proteins (Fig. 5A). These results support the conclusion that dark cells are engaged in a high level of POMC biosynthesis while light cells are biosynthetically quiescent, normally producing relatively little POMC.

Pharmacologic studies further support this conclusion. Subchronic haloperidol administration converted all light cells into darkly staining ones which, on morphometric analysis, exhibited a high density of protein synthesizing organelles (Fig. 5B). Thus, subchronic treatment had two effects on the intermediate pituitary, both of which independently increased the total production of POMC in the gland; it increased POMC mRNA levels in all cells and it stimulated biosynthetically quiescent light cells to produce high levels of POMC. Subchronic bromocriptine had the opposite effects on these two parameters. Together, these data suggest that light cells represent a reserve biosynthetic capacity of the intermediate lobe; normally 'on idle' they can be 'revved up' during periods of high demand.

We have also shown that longer term treatment (12-21 d) with dopaminergic agents produced yet another cellular response in the intermediate pituitary; chronic haloperidol stimulated and bromocriptine reduced the rate of cell proliferation in the gland. This

first became apparent when, during in situ hybridization experiments, we noticed that haloperidol treatment increased the number of cell layers in the intermediate lobe without affecting the size of individual cells. We then utilized (^{3}H)-thymidine uptake experiments and quantitation of the mitotic index to confirm that this hyperplastic response resulted from an increase in cell proliferation and not from selective cell death (Fig. 6).

These data show that activation or inhibition of cell surface receptors produces a much more complex and coordinated panoply of biochemical and cellular responses than previously anticipated. Acute haloperidol treatment stimulates rapid changes in POMC gene transcription and, hence, POMC biosynthesis. Subchronic treatment continues to accelerate POMC biosynthesis and also converts light cells, the biosynthetic reserve of the intermediate lobe, into dark cells, those actively engaged in synthesizing POMC, thus further expanding the biosynthetic capacity of the gland as a whole. Finally, after prolonged haloperidol treatment, the intermediate lobe exhibits cellular hyperplasia, an increase in the number of cells in the gland. Thus, the intermediate lobe has three independent, yet coordinated, mechanisms for modulating peptide production. Only after chronic treatment, however, do we observe selective changes in the activity of post-translational processing enzymes and concomitant alterations in β-endorphin processing. Thus, alterations in β-endorphin processing appear to be signal to the periphery that the organism is undergoing a persistent physiological stimuli, perceived by the central nervous system and communicated through the intermediate lobe.

2. Equine Cushing's disease: A model for prolonged changes in the dopaminergic regulation of the intermediate pituitary.

Millington, W.R., Dybdal, N.O., Dawson, R.D., Manzini, C. and Mueller, G.P. Equine Cushing's disease: Differential regulation of β-endorphin processing in tumors of the intermediate pituitary. Endocrinology 123:1598-1604, 1988.

Millington, W.R., Dybdal, N.O., Mueller, G.P. and Chronwall, B.M. N-acetylation and C-terminal proteolysis of β-endorphin in the anterior lobe of the horse pituitary. Gen. Comp. Endocrinology (Submitted).

The experimental results described above prompted us to consider whether the coordinated regulation of peptide acetyltransferase and POMC gene expression would persist if we continued the chronic administration of dopaminergic agents for longer periods of time. Unfortunately, treating rats for such prolonged periods is impractical. However, in certain species, including the dog and horse, the intermediate lobe spontaneously developes cellular hyperplasia, producing a Cushing's like syndrome (Moore, et al., 1979), morphologically quite similar to that produced pharmacologically in rats following chronic haloperidol treatment. Interestingly, the cause of equine Cushing's disease

has been attributed to the loss of hypothalamic dopaminergic neurons, similar to the chemical denervation we induce in rats with chronic haloperidol treatment.

Our initial experiments were designed to test the hypothesis that the development of equine Cushing's disease was, indeed, associated with a loss of dopaminergic innervation. To do so, we measured the concentrations of dopamine, serotonin and their respective metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) by HPLC (Kontur et al., 1984). We found that dopamine levels were reduced by a factor of ten in intermediate lobe tissue from Cushing's horses; DOPAC levels were completely undetectable (Table 2). Immunohistochemical studies confirmed the loss of tyrosine hydroxylase immunoreactive neurons within the hyperplastic tissue. Interestingly, levels of serotonin and 5-HIAA were not significantly different from age-matched control animals. These results show that, in equine Cushing's syndrome, the dopaminergic innervation of the intermediate lobe is almost completely lost while serotonergic neurons apparently remain intact. Thus, as in the case of the 'chemical denervation' produced by haloperidol, equine Cushing's syndrome is associated with a relatively specific loss of the dopaminergic regulation of the intermediate lobe.

Subsequent experiments showed that the intermediate lobe hyperplasia of equine Cushing's syndrome produces profound elevations in both plasma and CSF β-endorphin levels; β-endorphin was increased 60- and 120-fold in plasma and CSF, respectively. Nevertheless, there were no changes in either β-endorphin or POMC mRNA levels within the intermediate lobe itself. This indicates that, while the number of cells in the gland is substantially increased, the rate of POMC biosynthesis in individual intermediate lobe cells is unchanged.

We also found that the post-translational processing of β-endorphin was significantly altered in equine Cushing's syndrome relative to age matched controls. Specifically, there was proportionately more β-endorphin-1-31 and lower levels of N-acetylated and C-terminally shortened forms of the peptide (Fig. 7). This response is distinctly different from that observed following chronic haloperidol treatment although we have found comparable changes in β-endorphin processing in the absence of altered biosynthesis in rat brain following chronic haloperidol. This finding suggests either that β-endorphin processing enzymes are reduced in activity in equine Cushings's syndrome or that the duration of time between synthesis and secretion is reduced, leading to incomplete processing of the peptide. In either case, the data lend firm support to the concept that alterations in β-endorphin processing do occur in the absence of parallel changes in POMC biosynthesis. Moreover, these results indicate that, after prolonged alterations in the dopaminergic regulation of the intermediate lobe, cellular hyperplasia alone accounts for the increased peptide output of the gland; POMC gene transcription and the discrimination of light and dark cells has returned to control levels.

These experiments also revealed that β-endorphin is post-translationally processed to both N-acetylated and C-terminally shortened derivatives in the anterior lobe of the horse pituitary, a processing pattern qualitatively different from that of the rat and virtually every other mammalian species. Thus, separation of the molecular forms of β-endorphin using gel filtration and ion exchange chromatography showed that the horse anterior lobe primarily contains β-endorphin-1-31 and N-acetyl-β-endorphin-1-27 along with smaller amounts of β-lipotropin, β-endorphin-1-27 and N-acetyl-β-endorphin-1-31 and -1-26, in contrast to the rat anterior lobe, which contains approximately equal amounts of β-lipotropin and β-endorphin-1-31. Immunohistochemical experiments confirmed that N-acetyl-β-endorphin immunoreactivity is present in essentially all corticrophs in the horse, but not the rat. Gel filtration HPLC also demonstrated that β-lipotropin was almost entirely converted to β-endorphin-sized peptides, again unlike the rat in which approximately equal amounts of β-lipotropin and β-endorphin-1-31 are present. These data show that critical species differences occur in the distribution of peptide acetyltransferase and may potentially be useful in determining the factors which control the tissue specific expression of the enzyme as well as the functional role subserved by the tissue, and species, specific acetylation of β-endorphin peptides.

D. The Post-Translational Processing of β-Endorphin in Rat Brain:

Berglund, L.A., Millington, W.R. and Simpkins, J.W. Gonadal steroid and chronic morphine treatment do not change the post-translational processing of β-endorphin in rat brain. Life Sciences 44:591-601, 1989.

These studies were initiated to determine whether brain β-endorphin processing is regulated in a similar manner to that of the intermediate pituitary. Designing experiments to test this hypothesis is more difficult than for the pituitary, however, due to the relative paucity of information on the factors which regulated the activity of β-endorphin neurons. We examined three treatment paradigms; chronic haloperidol administration, morphine tolerance and dependence and gonadal steroid treatment following initial studies which evaluated the regional distribution of β-endorphin peptides in rat brain.

1. Regional heterogeneity in brain β-endorphin processing: Pronounced regional differences occur in brain β-endorphin processing. In the hypothalamus, which contains the majority of POMC cell bodies in brain, we found that β-endorphin-1-31 was the principal β-endorphin form, constituting 50 % of total immunoreactivity by ion exchange chromatography (Fig. 8). Approximately 25% of β-endorphin immunoreactivity is attributable to β-endorphin-1-27 and 18% coelutes with β-endorphin-1-26; acetylated β-endorphin peptides constitute less than 10% of total β-endorphin immunoreactivity. Previous studies have shown that this processing pattern is substantially different from that of the caudal medulla

which contains a second group of POMC perikarya. Here, β-endorphin-1-31 is a relatively minor product and N-acetylated, C-terminally shortened β-endorphin peptides predominate (Dores et al., 1986). Together, these data suggest that differing neuronal population utilize different β-endorphin processing pathways, much like the anterior and intermediate lobes of the pituitary.

We have also found that differences in β-endorphin processing occur among brain regions innervated by the hypothalamic POMC cell group. In the midbrain, the processing pattern is similar to that of the hypothalamus, but in the brainstem, β-endorphin-1-31 occurs in higher relative amounts, constituting 71% of total immunoreactivity, while β-endorphin-1-27 (17%) and β-endorphin-1-26 (10%) were relatively minor constituents. Differences in processing occur even within the hypothalamus itself; β-endorphin-1-31 is more extensively processed to C-terminally shortened forms in the medial basal hypothalamus than in the anterior hypothalamus/preoptic area. Heterogeneity in the post-translational processing of β-endorphin suggests that the regulation of β-endorphin processing enzymes is regionally selective.

2. The Regulation of β-endorphin processing in rat brain. During the initial year of this research, we examined the effect of chronic haloperidol treatment on the post-translational processing of β-endorphin in rat brain. This showed that β-endorphin processing was altered by haloperidol treatment in the midbrain; there was a higher proportion of β-endorphin-1-31 and lower levels of β-endorphin-1-27 and β-endorphin-1-26 (Fig. 9). Interestingly, these changes were not accompanied by elevated β-endorphin levels in the midbrain, hypothalamus, or in any of twelve other microdissected brain nuclei. Furthermore, the response appeared to be regionally specific, in that hypothalamic β-endorphin processing was not affected by haloperidol, perhaps because of regionally specific innervation by dopaminergic neurons. The observation that haloperidol altered midbrain β-endorphin processing without affecting POMC biosynthesis, indicates that regulation of brain β-endorphin processing clearly differs from that of the intermediate pituitary.

To further examine this hypothesis, we examined the effects of two additional pharmacologic treatment paradigms known to affect POMC biosynthesis; chronic morphine administration and gonadal steroid treatment. Chronic morphine renders rats, as well as human, tolerant and dependent to opiates; thus, any morphine induced changes in β-endorphin processing, that is, in the ratio of opiate agonist and antagonist forms of the peptide, could have important implications for the mechanism of opiate dependence. Earlier reports had shown that chronic morphine lowers both β-endorphin (Przewlocki et al., 1979) and POMC mRNA levels (Mocchetti et al., 1989) in rat brain and reduces the rate of β-endorphin release into the hypophysial portal vasculature (Koenig, J.I. et al., 1986), strong evidence that morphine inhibits the synthesis and release of hypothalamic β-endorphin.

Morphine dependence was induced in rats by implanting sustained release morphine pellets (75 mg/pellet) subcutaneously, one pellet day one of the experiment and two additional pellets on day three; the animals were killed on day five. We found, however, that morphine dependence did not produce any changes in the post-translational processing of β-endorphin in either the hypothalamus or periaqueductal grey, a region associated with the analgetic effects of morphine and β-endorphin (Fig. 10).

Estradiol treatment of ovariectomized rats lowers hypothalamic POMC mRNA (Wilcox and Roberts, 1985) and decreases β-endorphin levels in the hypothalamus and brain stem; progesterone treatment antagonizes the response (Wardlaw et al., 1982; Forman et al., 1985). These effects are of physiologic importance because the sequential administration of estradiol and progesterone to ovariectomized rats triggers the LH surge, a response thought to be mediated by a transient reduction in the tonic inhibitory control of LH release maintained by hypothalamic β-endorphin neurons (Gabriel et al., 1986). The LH surge is also associated with a refractoriness to exogenous opiates, which indicates that inhibition of β-endorphin synthesis and release does not fully account for the response. We hypothesized that this desensitization could result from a steroid induced change in the enzymatic processing of β-endorphin, producing a higher proportion of opiate antagonist β-endorphin-1-27 and lower levels of opiate agonist β-endorphin-1-31. This was an ideal paradigm for examining the regulation of β-endorphin processing because a well characterized physiologic output, the LH surge, could be correlated with changes in processing. Furthermore, clear evidence was available that gonadal steroids lower the rate of POMC biosynthesis and, in ther intermediate pituitary, we knew that alterations in POMC synthesis were associated with distinct changes in the post-translational processing of β-endorphin.

We found, however, that gonadal steroid treatment had no effect on the post-translational processing of β-endorphin in rat brain. There were no significant differences in β-endorphin processing between controls and rats treated with either estradiol alone or estradiol plus progesterone in either the preoptic area of the hypothalamus, the medial basal hypothalamus or the brainstem. Thus, the LH surge is not associated with altered β-endorphin processing. Moreover, these results demonstrate that changes in POMC biosynthesis in brain, unlike the intermediate pituitary, do not produce parallel changes in β-endorphin processing.

To summarize, we have examined the effects of three different treatment paradigms on β-endorphin processing in rat brain; chronic haloperidol, morphine tolerance and dependence, and gonadal steroids. Only the first, haloperidol treatment, altered processing but this was not associated with changes in POMC biosynthesis. Morphine and gonadal steroid treatments, which do alter POMC synthesis, do not affect β-endorphin processing. These results indicate that β-endorphin processing in brain is regulated differently than in the intermediate pituitary and is not affected by

treatments which alter POMC synthesis. Additional, yet to be identified, mechanisms appear to regulate brain β-endorphin processing.

E. The Post-Translational Processing of β-Endorphin in Human Brain:

Millington, W.R. and Smith, D.L. The post-translational processing of β-endorphin in human hypothalamus. J. Neurochem. (In Press).

The presence of β-endorphin immunoreactivity in human brain was first demonstrated soon after the initial discovery of the peptide (Gramsch et al., 1980; Emson, et al., 1984). Its distribution is similar to that of the rat and other mammals and, like the rat, most of the immunoreactive β-endorphin in human brain is localized within neuronal processes arising from cell bodies in the medial basal hypothalamus (Bugnon, et al., 1979). However, exactly how β-endorphin is processed in the human, whether to opiate active or inactive forms, is completely unknown. This information is essential for predicting whether the physiological effects and regulatory mechanisms we observed in the rat are relevant to the human. Moreover, our long term objective of developing clinically useful pharmacologic strategies for modifying brain β-endorphin processing demands that the animal species we study accurately models the human β-endorphin processing pathway. There is reason to believe that this may not be the case, however, because the primary structure of human β-endorphin differs in certain critical respects from the rat and virtually all other mammalian species (Li, 1984).

Before initiating these experiments we addressed a problem common to studies of human autopsy material; the effect of the postmortem interval, that is, the duration of time between death and freezing the tissue. To test whether the β-endorphin processing pattern is artifactually changed during the postmortem interval, we conducted experiments with rats which mimicked the treatment conditions for human autopsy material. We found that storing rat hypothalami, either at room temperature for 8 hours or at 4°C for 2, 6, 12 or 24 hours, had no effect whatsoever on the molecular forms of β-endorphin (Smith and Millington, 1989). Significant decrements did occur, however, in total β-endorphin immunoreactivity following storage at 4°C for six hours or longer. These results indicate that while some β-endorphin is lost during the postmortem interval, the overall processing pattern still reflects that present immediately after death.

Having established the feasibility of using human tissue, we proceeded to examine the molecular forms of β-endorphin in human hypothalamus. The samples were provided to us by the Brain Tissue Resource Center of McLean Hospital, an affiliate of Harvard Medical School. The subject population consisted of both males and females ranging in age from 23 to 89 years with no history of psychiatric or neurologic disease. β-endorphin peptides were isolated by

cation exchange high performance liquid chromatography (HPLC) and β-endorphin immunoreactivity was measured in the chromatography fractions by radioimmunoassay. This revealed that β-endorphin-1-31 was the principal form, constituting 56.2 ± 5.3 percent of total immunoreactivity (Fig. 11). β-endorphin-1-27 (16.6 ± 2.0%) and β-endorphin-1-26 (15.0 ± 2.1%) were also present but acetylated forms were quantitatively minor, each comprising approximately 5% of total β-endorphin. As one might predict, β-endorphin processing varied among individual samples, some containing relatively high levels of acetylated β-endorphin peptides, but these differences did not appear to be correlated with age, sex, cause of death or any other identifiable parameter. We also identified the β-endorphin forms in subregions of the hypothalamus, including the preoptic and suprachiasmatic nuclei, and found the processing pattern in these areas to be similar to the whole hypothalamus, suggesting that regional differences do not occur within the hypothalamus. Efforts to isolate β-endorphin peptides from the amygdala, nucleus accumbens, median eminence and cerebrospinal fluid were not successful owing to the very low levels of the peptide in these tissues. Preliminary experiments revealed no differences in hypothalamic β-endorphin processing between control and schizophrenic subjects.

These studies are the first to identify the β-endorphin processing pathway in human brain. The results show that, despite important differences in primary sequence, β-endorphin is processed similarly in both human and rat hypothalamus (Zakarian and Smyth, 1979; Emeson and Eipper, 1986; Berglund et al., 1989), thereby confirming that the rat is an appropriate model for studying the regulation of brain β-endorphin processing. They also provide evidence that the same, or similar enzymes process β-endorphin in both species. As in the rat, C-terminal proteolysis is the primary processing pathway in human hypothalamus; however, the predominance of N-acetylated β-endorphin peptides in rat brainstem, hippocampus, colliculae (Zakarian and Smyth, 1979), nucleus accumbens (Dennis, et al., 1983) and caudal medulla (Dores et al., 1986) raises the possibility that N-acetylation is the primary pathway in the corresponding human brain regions as well. These findings provide further support for the concept that chemical agents designed to alter the activity of β-endorphin processing enzymes will have important effects on the physiology of brain β-endorphin and may be of significant clinical utility.

F. Central Cardiovascular Effects of β-Endorphin Peptides:

Hirsch M.D. and Millington, W.R. Endoproteolytic conversion of β-endorphin-1-31 to β-endorphin-1-27 potentiates its central cardioregulatory activity. Brain Research (In Press).

One of the most intriguing aspects of β-endorphin processing is that relatively minor post-translational changes in peptide structure produce profound changes in biological activity. As discussed previously, the potent analgetic properties of β-endor-

phin-1-31 are essentially abolished by both N-acetylation and C-terminal proteolysis. Moreover, in addition to substantially reducing agonist activity, C-terminal proteolysis produces an entirely different property; it converts β-endorphin-1-31 from an agonist to a highly potent opioid receptor antagonist, β-endorphin-1-27 (Nicolas and Li, 1985). Receptor binding studies also showed that β-endorphin-1-27 displaces both etorphine and β-endorphin-1-31 binding, further evidence that the peptide functions as an antagonist at mu and/or delta opioid receptors (Akil et al, 1981; Nicolas and Li, 1985). These intriguing findings prompted us to raise a broader, rather fundamental question; does β-endorphin-1-27 block other physiologic and behavioral responses mediated by β-endorphin neurons or is the relationship between processing and biological activity functionally specific? To address this question we initiated studies of the role of β-endorphin processing in the central regulation of cardiovascular function.

To study β-endorphin's central cardioregulatory effects, we administered β-endorphin-1-31 or -1-27 intracisternally (ic) to chloralose-anesthetized rats and recorded mean arterial pressure (MAP), pulse pressure and heart rate directly through an arterial cannula. Our initial experiments demonstrated, as others had shown previously, that β-endorphin-1-31, like morphine, lowers blood pressure (Petty and de Jong, 1982; Holaday, 1983). MAP was reduced by approximately 30 mm Hg 60 min after ic injection of 1.5 nmol β-endorphin-1-31 and returned toward baseline values by 120 min (Fig. 12). Unexpectedly, β-endorphin-1-27 also produced a potent hypotensive response; indeed, its potency was 5-10 fold greater than β-endorphin-1-31 (Hirsch et al., 1988). Thus, β-endorphin-1-27 (1.5 nmol) lowered MAP to a significantly greater extent than an equimolar β-endorphin-1-31 dose while 0.15 nmol β-endorphin-1-27 produced a response essentially equivalent to 1.5 nmol of the parent peptide (Fig. 12; Table 4). β-endorphin-1-27 also produced bradycardia but only at the higher dose tested (1.5 nmol); neither β-endorphin-1-31 nor the lower dose of β-endorphin-1-27 had any effect on heart rate. Respiration was not affected by either peptide. Thus, C-terminal proteolysis potentiates the hemodynamic actions of β-endorphin while greatly reducing its analgetic potency, converting it to an opioid receptor antagonist. This clearly demonstrates that post-translational processing subserves quite different roles in defining the analgetic and cardioregulatory responses produced by β-endorphin peptides.

Next, we examined the hemodynamic potency of the other four β-endorphin forms, β-endorphin-1-26 and N-acetyl-β-endorphin-1-31, -1-27 and -1-26. This revealed that all four peptides were completely devoid of either hypotensive or bradycardic activity (Fig. 13). Thus, while the conversion of β-endorphin-1-31 to -1-27 enhances hemodynamic potency, both N-acetylation and further C-terminal proteolysis to β-endorphin-1-26 inactivate the peptide. These processing steps therefore produce the same effect on both analgesia and hypotension; they eliminate bioactivity.

Finally, we tested whether the hemodynamic action of β-endorphin peptides could be inhibited with the opioid receptor antagonist, naloxone. We found that indeed it was; naloxone pretreatment (300 nmol, ic) completely blocked the effects of both β-endorphin-1-31 and β-endorphin-1-27 on MAP and heart rate but produced no hemodynamic effects when given alone (Table 4). This indicates that the cardioregulatory effects of both peptides are mediated by opioid receptors.

Clearly, C-terminal proteolysis is a critical determinant of the pharmacological activity spectrum of β-endorphin peptides. However, to demonstrate the physiologic relevance of these pharmacologic data, it is essential to determine whether these pharmacologically active β-endorphin forms are actually present in brain. We therefore characterized the molecular forms of β-endorphin present in the nucleus of the solitary tract (nTS), an area importantly involved in the central regulation of cardiovascular function (Holaday, 1983). The nTS β-endorphin processing pathway is of particular interest because a relatively dense population of β-endorphin containing neuronal perikarya are localized within the nucleus; indeed, it is the only brain region outside of the medial basal hypothalamus where endorphinergic cell bodies are found (Joseph et al., 1983; Pilcher and Joseph, 1986; Palkovits et al., 1987). Furthermore, microinjection of β-endorphin-1-31 directly into the nTS lowers blood pressure (Petty and de Jong, 1982), suggesting that exogenously administered β-endorphin peptides act through receptors normally activated by β-endorphin released from nTS neurons. To examine the β-endorphin processing pathway in the nTS, we microdissected the nucleus using the Palkovits technique (Palkovits et al., 1987), pooling tissue from approximately one hundred animals, and separated β-endorphin peptides by cation exchange chromatography (Millington et al., 1987). This revealed that β-endorphin-1-31 is the quantitatively major form in the nTS, constituting 52% of total immunoreactivity. Relatively large amounts of β-endorphin-1-27 (21%) were also present but N-acetyl-β-endorphin-1-31 (5%), -1-27 (13%) and -1-26 (8%) were relatively minor products. These results show that the forms of β-endorphin which produce hypotension when injected in pharmacologic doses are, indeed, synthesized in the nTS.

The mechanism responsible for the differential analgetic and cardioregulatory effects of β-endorphin-1-27 remains to be established but the data are consistent with evidence that β-endorphin peptides exert their diverse effects by activating several different opioid receptor subtypes. The analgetic activity of β-endorphin-1-31 is thought to be mediated by mu and/or delta opioid receptors (Pasternak, 1987) and there is strong evidence that β-endorphin-1-27 also acts by blocking these same receptor subtypes (Nicolas and Li, 1985). Additional evidence further suggests, however, that certain β-endorphin induced responses are mediated by a third opioid receptor subtype, the epsilon receptor. Data supporting this concept is derived, in part, from receptor binding experiments using brain tissue (Goodman et al., 1983; Houghten et al., 1984), but also from physiological studies of peripheral

tissues, including the rat vas deferens and guinea pig ileum, that have served as model systems for opiate research for many years (Schulz et al., 1981; Huidobro-Toro et al., 1982; McKnight et al., 1983). These studies showed that β-endorphin-1-27 acts as an agonist at epsilon receptors; indeed, it's potency is, in some studies, even greater than that of β-endorphin-1-31. Furthermore, epsilon receptors are also blocked by naloxone. These properties are consistent with our studies of the hemodynamic effects produced by β-endorphin-1-27, suggesting that the peptide may produce hypotension by activating receptors similar to the putative epsilon opioid receptor. The differential effects of β-endorphin peptides on analgesia and cardiovascular regulation thus support the hypothesis that a single processing step, C-terminal proteolysis, produces entirely different changes in biological activity depending upon the postsynaptic receptors which mediate the response.

SUMMARY AND CONCLUSIONS

1. Co-Regulation of Peptide Acetyltransferase, POMC Biosynthesis and β-Endorphin Processing in the Intermediate Pituitary: The results of these studies clearly establish the feasibility of selectively controlling the biosynthesis of β-endorphin peptides using chemical agents targeted on cell surface receptors to modify the activity of post-translational processing enzymes. We found that post-translational processing enzymes are selectively regulated and that regulation of these enzymes leads to specific and predictable changes in the biological activities of the peptides they synthesize (Millington et el., 1986, 1987) Thus, in the intermediate pituitary, peptide acetyltransferase activity is induced by treatments which accelerate POMC biosynthesis. The coordinate regulation of peptide acetyltransferase activity and POMC biosynthesis appears to be a mechanism for maintaining the extent of acetylation of β-endorphin peptides independent of changes in the secretory activity of the gland.

Carboxypeptidase H, the enzyme which converts β-endorphin-1-27 to β-endorphin-1-26, is not induced, however, leading to specific changes in the precursor to product ratio of these two peptides. Thus, post-translational processing enzymes appear to be selectively regulated. Comparison of their relative activities reveals why this is so; the Vmax of carboxypeptidase H is several orders of magnitudes higher than peptide acetyltransferase (Millington and Chronwall, 1989). Thus, carboxypeptidase H does not appear to be a regulatory enzyme according to the three criteria we established earlier; it has a high Vmax, it is not inducible and it is substrate driven; its usual substrates, peptides with C-terminally extended lysine or arginine residues, are immediately converted to their respective peptide products. The one exception, removal of the C-terminal histidine from β-endorphin-1-27, results from the exceedingly low affinity of carboxypeptidase H for histidine residues. Peptide acetyltrans-

ferase, on the other hand, satisfies all the criteria for an enzyme which serves a regulatory role; it has a low Vmax, it is inducible and high levels of both substrates and products are present in the intermediate lobe and brain.

The ultimate significance of changes in β-endorphin processing remain to be established, however. For the endocrine POMC system evaluating the significance of altered β-endorphin processing depends upon a definitive understanding of the physiologic function of the intermediate pituitary as well as the identification of the individual target sites of the multiple molecular forms of β-endorphin released from the intermediate lobe into the circulation. While the physiological function of the intermediate pituitary is not completely understood, one of its most important roles appears to involve mediating the response of the organism to stressful stimuli. Previous studies from this and other laboratories (Muller, 1981; Berkenbosch et al., 1984) have shown that stress, whether produced by surgery, pain, immobilization or changes in ambient temperature, is the most potent stimulus known for releasing β-endorphin from the intermediate lobe. Furthermore, stress releases β-endorphin both by activating neuronal pathways projecting from the hypothalamus and by elevating circulating epinephrine released by sympathetic activation of the adrenal medulla (Berkenbosch et al, 1984). Thus, the intermediate pituitary appears to function as a neuroendocrine transducer system, integrating both neural and autonomic inputs to produce a hormonal output, β-endorphin and other POMC-derived peptides, which transmit chemically encoded information regarding stressful stimuli to the periphery.

What then are the ultimate target sites of the β-endorphin peptides released from the intermediate pituitary? The quantitatively major forms of β-endorphin in the intermediate lobe lack affinity for opioid receptors; however, recent studies have identified several distinct classes of non-opioid β-endorphin binding sites possessing varying specificities for the N-terminal (Matthews et al., 1984), C-terminal (Hazum et al., 1979; Schweigerer et al., 1983) or mid-region (Dave, et al., 1985) sequences of the peptide, in peripheral tissues and on cellular elements of the immune system. Indeed, differing β-endorphin binding sites in the immune system appear to discriminate among very small changes in the peptide's sequence. This suggests that β-endorphin peptides released from the intermediate lobe may play a role in mediating the immune response to stress. Communications between the central nervous system and elements of the immune system by means of neuroendocrine peptides is currently an area of intensive investigation.

In light of these findings, we hypothesize that selective alterations in β-endorphin processing function as a signal to the periphery that the organism is experiencing a persistent stressful stimuli. This hypothesis is supported by the results of studies by Akil et al. (1985) who found that chronic, but not acute, stress differentially altered β-endorphin processing, producing changes

biosynthesis and processing similar to those we observed following chronic dopaminergic antagonist treatment. Their results clearly show that long term stress alters the molecular forms and, hence, the bioactivities of β-endorphin peptides. The selective regulation of post-translational processing enzymes thus provides a mechanism for encoding information about the temporal nature of environmental stimuli perceived by the central nervous system and transmitted to the immune system and other peripheral tissues by the intermediate pituitary.

2. The Regulation of β-Endorphin Processing in Rat Brain: Our results also show that neuronal β-endorphin processing is not fixed but can be modified by chemical agents which interact with cell surface receptors, such as haloperidol. The effects of haloperidol appear to be independent of changes in β-endorphin biosynthesis, however, indicating that the regulation of brain β-endorphin processing differs from that of the intermediate pituitary. The specific effects of haloperidol were intriguing; it increased the proportion of opioid active β-endorphin-1-31 and reduced levels of the opioid receptor antagonist and inactive forms. Furthermore, its effects were regionally selective, altering β-endorphin processing in the midbrain, but not in the hypothalamus. Nevertheless, treatments which affect POMC gene expression in brain, such as chronic morphine and gonadal steroid administration, have no effect on brain β-endorphin processing whereas, in the intermediate lobe, changes in POMC biosynthesis are accompanied by specific alterations in the molecular forms of β-endorphin released from the gland. This indicates that additional, yet to be identified, mechanisms regulate peptide processing in brain. Moreover, we have concluded from these studies that brain β-endorphin processing is relatively resistant to modifications by chemical agents targeted on cell surface receptors. This empha-sizes the need to develop pharmacologic strategies for directly inhibiting, or activating, the enzymes which post-translationally process β-endorphin.

3. The Post-Translational Processing of β-Endorphin in Human Brain: The presence of β-endorphin immunoreactivity in human brain was demonstrated over a decade ago but exactly how the peptide is processed, whether to opioid active or inactive forms, is completely unknown. This information is essential to establish whether studying the function and regulation β-endorphin processing in rat brain is applicable to human neurobiology. Our studies first established that artifactual changes in processing do not occur during the post-mortem interval by conducting experiments with rats which mimicked the treatment conditions for human autopsy material. We then isolated β-endorphin peptides from human hypothalamus using cation exchange HPLC. This revealed that opioid active β-endorphin-1-31 was the quantitatively major form, although significant amounts of β-endorphin-1-27 and -1-26 are also present (Millington and Smith, 1991, in press). N-acetyl β-endorphin peptides were quantitatively minor, together constituting approximately 15% of total immunoreactivity. A similar processing pattern

was found in the preoptic and suprachiasmatic nuclei. These results demonstrate that, despite important differences in primary structure, β-endorphin is processed similarly in both human and rat hypothalamus, thereby confirming that the rat is an appropriate model for investigating the function and regulation of β-endorphin processing.

4. Central Cardiovascular Effects of β-endorphin Peptides: These studies demonstrated that post-translational processing defines the central cardioregulatory actions of β-endorphin. As shown previously (Petty and de Jong, 1982), β-endorphin-1-31, like morphine, is a potent hypotensive agent when administered centrally. β-endorphin-1-27, also lowers blood pressure; indeed its potency is 5-10 fold greater than β-endorphin-1-31 (Hirsch and Millington, 1991, in press). This relationship is in marked contrast to analgesia where C-terminal shortening abolishes agonist activity, converting β-endorphin-1-31 from a highly potent agonist to an opioid receptor antagonist, β-endorphin-1-27 (Nicolas and Li, 1985). However, both further C-terminal proteolysis of β-endorphin-1-27 to β-endorphin-1-26 and N-acetylation have the same effect on analgesia and hypotension; they abolish bioactivity. These findings indicate that post-translational processing can produce either the same, or entirely different changes in peptide bioactivity depending upon the postsynaptic receptors which mediate the response. Thus, both pre- and post-synaptic mechanisms control the physiologic effects produced by β-endorphin neurons: pre-synaptic mechanisms regulate β-endorphin processing enzymes, producing different bioactive β-endorphin forms; post-synaptic mechanisms regulate the expression of opioid receptor subtypes, producing different responses to the same β-endorphin form.

PUBLICATIONS

This research support resulted in the publication, or submission for publication, of twelve manuscripts and nineteen abstracts. One additional manuscript is currently being written and two remain to be prepared, with research still in progress.

Manuscripts:

Millington, W.R., O'Donohue, T.L., Chappell, M.C., Roberts, J.L. and Mueller, G.P. Coordinate regulation of peptide acetyltransferase activity and proopiomelanocortin gene expression in the intermediate lobe of the rat pituitary. Endocrinology 118:2024-2033, 1986.

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Lavigne, G.L., Millington, W.R. and Mueller, G.P. The CCK-A and CCK-B antagonists, devazepide and L-365,260, enhance morphine antinociception only in non-acclimated rats. Pain (Submitted).

Millington, W.R., Mueller, G.P. and Lavigne, G.L. Differential effects of cholecystokinin receptor type A and B antagonists on cholecystokinin-stimulated pituitary β-endorphin secretion. (In preparation).

Abstracts:

Chronwall, B.M., Millington, W.R., Hook, G.R. and O'Donohue. T.L. Histological evaluation of the dopaminergic regulation of POMC gene expression in the intermediate lobe of the rat pituitary. International Congress of Pharmacology, 1987.

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PERSONEL

The following personel received salary support from this research program:

William R. Millington, Ph.D.: Principal Investigator. The PI received full salary support between February 1, 1986 and May 31, 1989. The PI is currently Associate Professor of Molecular Biology and Biochemistry, University of Missouri-Kansas City, Kansas City, MO. This research grant support resulted in two successful grant applications:

USAMRDC (DAMD17-90-Z-0022) "Glycyl-L-glutamine: A dipeptide neurotransmitter derived from β-endorphin".

National Institute on Drug Abuse (DA04598) "The post-translational processing of β-endorphin"

Michael D. Hirsch, Ph.D.: Research Assistant Professor. Dr. Hirsch received full salary support between June 1, 1987 and April 30, 1989 and between September 1, 1989 and June 30, 1989, during which time he served as Principal Investigator. This research support enabled Dr. Hirsch to establish independent funding from the National Science Foundation. He now serves as Executive Secretary to the Cellular Neurobiology Study Section, Neuroscience Research Review Branch, National Institute of Mental Health, ADAMHA.

Debra L. Smith: Debra Smith received full salary support, as an undergraduate summer employee, between June 1 and August 31, 1987 and again between June 1 and August 31, 1988. She presented her research at a national meeting and is co-author of a manuscript, now in press. Debra is currently a medical student at Johns Hopkins University.

Laura E. Davidow: Laura Davidow received full salary support, as an undergraduate summer employee, between June 5 and August 31, 1988. She is currently a medical student at the University of Michigan.

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Table 1. The effect of chronic haloperidol on the kinetic parameters of intermediate lobe peptide acetyltransferase.

Substrate	Treatment	
	Control	Haloperidol
Des-Acetyl-α-MSH		
Vmax (pmol/min/mg prot)	80.7 ± 16.6	154.7 ± 4.8
Km (μM)	24.3 ± 1.1	32.3 ± 2.6
Acetyl Coenzyme A		
Vmax (pmol/min/mg prot)	88.0 ± 15.0	143.7 ± 17.8
Km (μM)	7.1 ± 1.9	6.8 ± 1.3

Table 2. Equine Cushing's disease depletes intermediate lobe dopamine and DOPAC, but not serotonin or 5-HIAA concentrations.

	Dopamine	DOPAC	Serotonin	5-HIAA
Control	23.2 ± 3.8	8.0 ± 1.8	11.0 ± 1.9	2.5 ± 0.5
Cushing's	2.7 ± 0.8*	ND	15.3 ± 5.6	3.0 ± 0.7

Dopamine, DOPAC, serotonin and 5-HIAA were analyzed by HPLC with electrochemical detection. The data are expressed as the mean ± SE of five to nine subjects in each group, expressed as picomoles per mg protein, and were analyzed by two-tailed t test. ND = not detectable. * $P < 0.05$ differs from control

Table 3. The post-translational processing of β-endorphin in human hypothalamus.

Case Number	β-Endorphin Peptide (percent total immunoreactivity)					
	Ac-β-1-26	Ac-β-1-27	β-1-26	β-1-27	Ac-β-1-31	β-1-31
1	1.7	1.5	13.7	18.5	2.4	62.2
2	5.7	7.0	35.5	32.9	0	11.0
3	10.0	12.0	14.6	12.0	15.0	36.5
4	5.4	6.1	20.1	9.5	10.9	48.1
5	7.7	0	14.7	13.5	2.0	62.1
6	9.2	8.4	11.1	14.9	6.0	50.5
7	0	2.9	6.8	10.3	2.3	77.6
8	0.4	1.6	10.6	14.9	0.4	69.1
9	1.0	3.5	8.1	11.8	0	75.7
10	2.5	0	12.3	16.2	1.8	67.2
11	1.8	6.3	15.7	25.2	5.6	58.4
12	2.7	3.4	17.1	19.8	1.0	56.0
	4.0 ± 1.0	4.4 ± 1.1	15.0 ± 2.1	16.6 ± 2.0	4.0 ± 1.4	56.2 ± 5.3

Human hypothalami were homogenized in 1 N acetic acid, concentrated by Sep-Pak extraction and β-endorphin peptides were separated by cation exchange high performance liquid chromatography (HPLC) and analyzed by radioimmunoassay. The data are presented as the per cent of total immunoreactivity eluted from the cation exchange column for each individual sample. Ac = N-acetyl; β = β-endorphin.

Table 4. Effects of β-endorphin peptides on cardiovascular regulation.

PEPTIDE	DOSE (nmol)	MAP (mm Hg)	HEART RATE (beats/min)
β-END-1-31	1.50	-29.7 ± 3.9**	+17.8 ± 21.4
+ NALOXONE		-4.7 ± 4.7	-4.0 ± 25.0
β-End-1-27	1.50	-47.3 ± 5.3**	-78.6 ± 17.2*
	0.15	-25.9 ± 4.7**	-12.6 ± 12.8
+ NALOXONE		-3.0 ± 2.0	+41.2 ± 15.3
β-END-1-26	1.50	-2.3 ± 1.3	+4.4 ± 8.0
Ac-β-END-1-31	1.50	+0.3 ± 5.0	+9.8 ± 11.4
Ac-β-END-1-27	1.50	+0.1 ± 3.2	+31.8 ± 10.8
Ac-β-END-1-26	1.50	+0.4 ± 1.6	+4.4 ± 3.3

Mean arterial pressure (MAP) and heart rate were measured through an arterial catheter in anesthetized rats. β-endorphin peptides or CSF were injected i.c. at the indicated dose. Naloxone HCl (300 nmol, i.c.) was administered 30 min prior to peptide treatments. The data are presented as the mean difference ± S.E. in MAP and HR between baseline pretreatment values and those recorded 60 min following peptide administration (N = 5 per group) and were analyzed by ANOVA (unweighted means) followed by Dunnett's t tests. End = endorphin; Ac = acetyl.
* $P < 0.05$; ** $P < 0.01$ differs from control.

FIGURE LEGENDS

Figure 2. The effect of haloperidol or bromocriptine treatment on POMC mRNA, peptide acetyltransferase activity, and α-MSH immunoreactivity in the intermediate pituitary. Groups of six to eight rats were treated with haloperidol (2 mg/kg/d), bromocriptine (4 mg/kg/d) or vehicle (20 mM tartaric acid) for 21 days and were sacrificed 24 h following the final drug treatment. The letters beneath the ordinate identify the treatment groups (C, control; H, haloperidol; B, bromocriptine) The data are expressed as the mean ± SE and statistical differences were determined by two-tailed t test. * $P < 0.05$; ** $P < 0.01$ differs from control.

Figure 3. Intermediate lobe carboxypeptidase H activity is not induced by chronic haloperidol administration. Groups of eight rats were treated for 21 days with haloperidol (2.0 mg/kg/d) or vehicle and carboxypeptidase H activity was assayed by radiometric assay. * $P < 0.05$ differs from control.

Figure 4. Effects of chronic haloperidol or bromocriptine administration on intermediate lobe β-endorphin processing. Groups of eight rats were treated with either haloperidol (2.0 mg/kg), bromocriptine (4.0 mg/kg) or vehicle for 21 days. β-Endorphin peptides were separated by cation exchange chromatography. The peaks of immuno-reactive β-endorphin are: (I) N-acetyl-β-endorphin-1-26; (II) N-acetyl-β-endorphin-1-27; (III) β-endorphin-1-27; (IV) N-acetyl-β-endorphin-1-31; and (V) β-endorphin-1-31.

Figure 5. Morphometric analysis of light and dark cells in the rat intermediate lobe. Panel A: Morphometric analysis of rough endoplasmic reticulum (RER) and mitochondria in light and dark staining melanotrophs. Panel B: The effects of subchronic haloperidol or bromocriptine administration on the ratio of light to dark staining melanotrophs. * $P < 0.05$; ** $P < 0.01$ differs from control.

Figure 6. The effects of chronic haloperidol or bromocriptine treatments on [^{3}H]-thymidine uptake in the rat intermediate lobe. Groups of five rats were treated with haloperidol (H; 10 mg) bromocriptine (B; 10 mg) or placebo (C) pellets implanted subcutaneously and were killed after 12 days of treatment. [^{3}H]-thymidine (2 μCi/g) was administered 2 h before the animals were killed. The data are expressed as the mean ± SE and were analyzed by ANOVA followed by Duncan's multiple range test. * $P < 0.05$ vs. control.

Figure 7. The molecular forms of β-endorphin in the intermediate lobe of control horses and tumor tissue from horses with Cushing's disease. β-endorphin peptides were separated from pooled extracts of 11 control and 8 Cushing's horses by cation exchange chromatography. The arrows mark the elution positions of: (I) N-acetyl-β-endorphin-1-26; (II) N-acetyl-β-endorphin-1-27; (III) β-endorphin-1-27; (IV) N-acetyl-β-endorphin-1-31; and (V) β-endorphin-1-31.

Figure 8. The post-translational processing of β-endorphin in rat hypothalamus. β-endorphin peptides were separated by cation exchange chromatography from extracts of 8 rat hypothalami.

Figure 9. The effect of chronic haloperidol administration on β-endorphin processing in rat midbrain. Groups of eight rats were treated with haloperidol (2 mg/kg/d) for 21 days and β-endorphin were separated from midbrain extracts by cation exchange chromatography.

Figure 10. Chronic morphine administration does not affect β-endorphin processing in rat hypothalamus (left panels) or periaqueductal gray (right panels). Groups of five rats received morphine (75 mg) or placebo pellet implants, one on day one followed by two additional pellets 48 h later, and were killed four days thereafter. β-endorphin peptides were separated by cation exchange chromatography. Left panels (hypothalamus): (I) β-endorphin-1-26; (II) β-endorphin-1-27; (III) β-endorphin-1-31. Right panels (periaqueductal gray): (I) β-endorphin-1-26; (II) β-endorphin-1-27; (III) N-acetyl-β-endorphin-1-31; (IV) β-endorphin-1-31

Figure 11. The post-translational processing of β-endorphin in human hypothalamus. Human hypothalami were homogenized in 1 N acetic acid, β-endorphin peptides were extracted using a Sep-Pak cartridge (Waters Corp.) and separated by cation exchange HPLC.

Figure 12. C-terminal proteolysis of β-endorphin-1-31 enhances hemodynamic potency. Groups of 5 rats were anesthetized with chloralose and mean arterial pressure (MAP) was recorded at five minute intervals using a Gould model 30-V8202-10 physiograph equipped with a pressure transducer attached to a tail artery cannula. β-endorphin peptides were injected intracisternally (ic) dissolved in 5 μl artificial cerebrospinal fluid.

Figure 13. N-acetylation and C-terminal proteolysis of β-endorphin-1-27 eliminates hemodynamic activity. MAP was recorded at five minute intervals for ninety minutes as described in figure 12. The data represent the mean ± SE (n = 5) recorded sixty minutes after peptide administration.

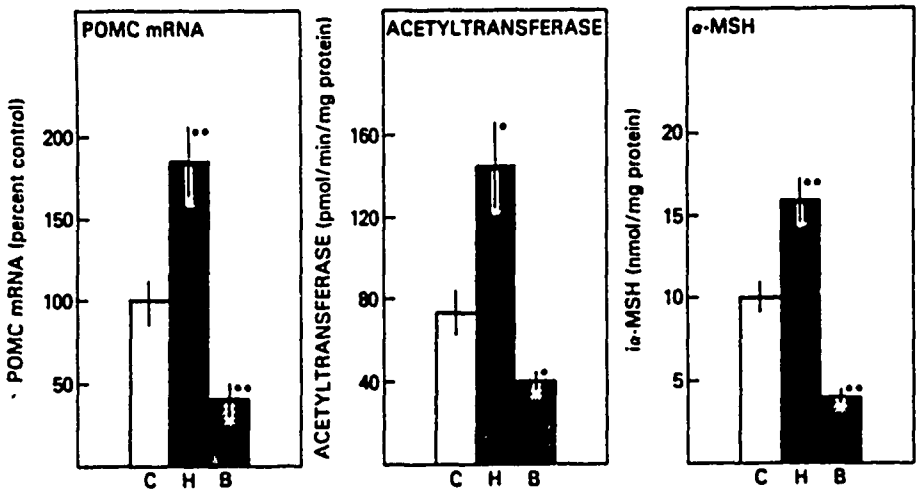


FIGURE 2

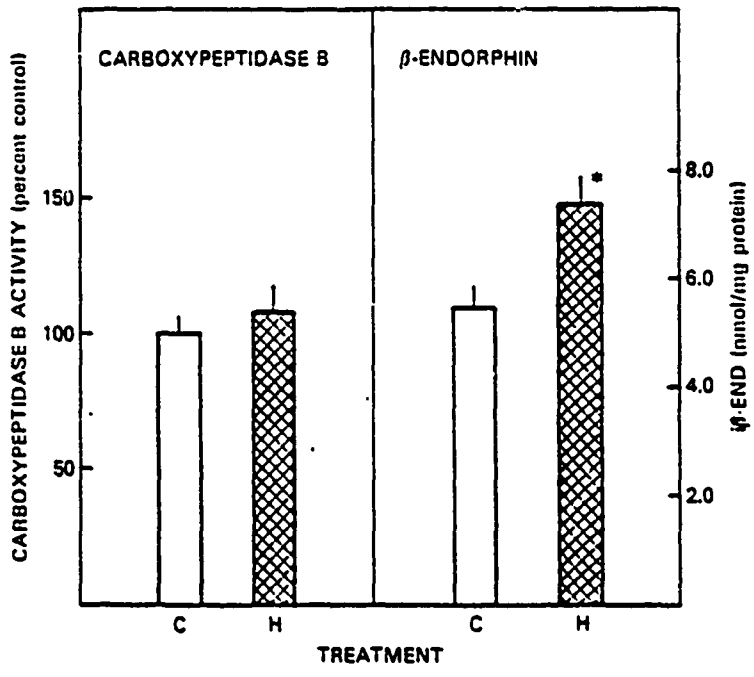
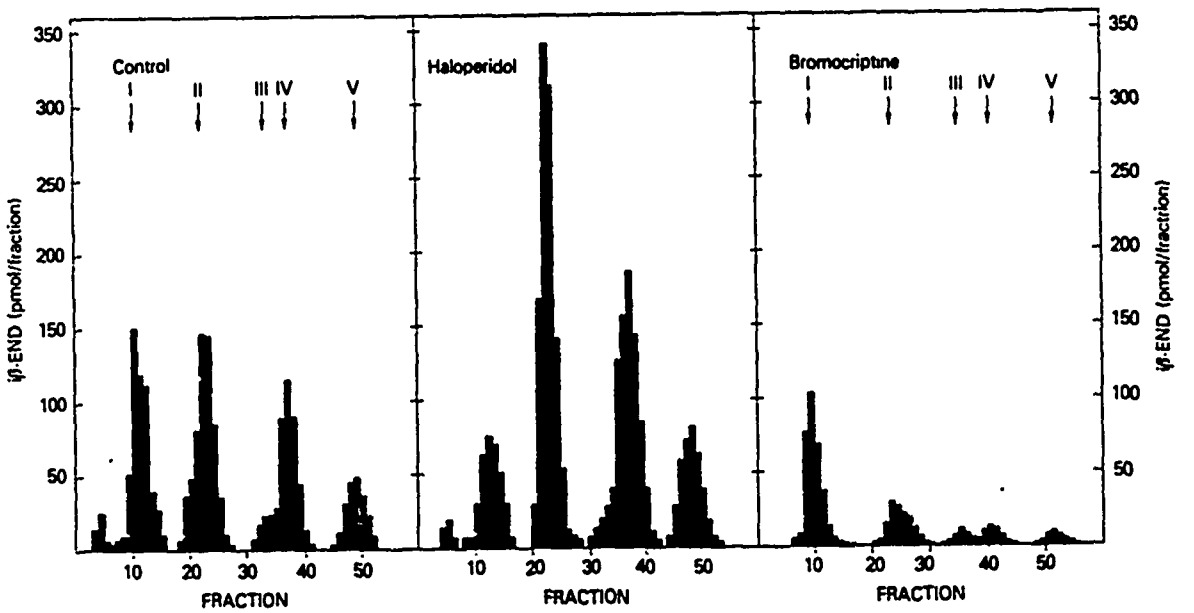


FIGURE 3



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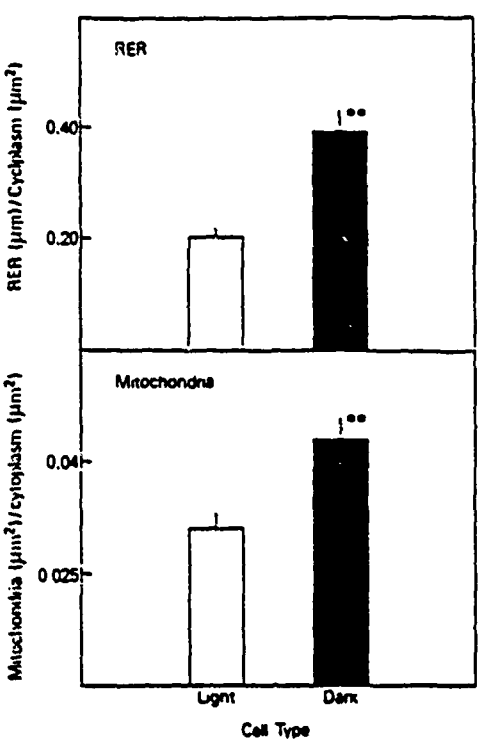


FIGURE 5A

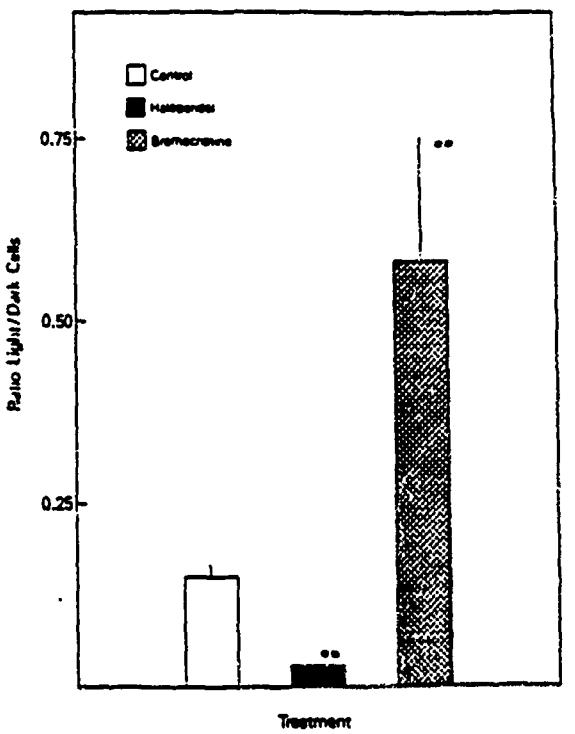


FIGURE 5B

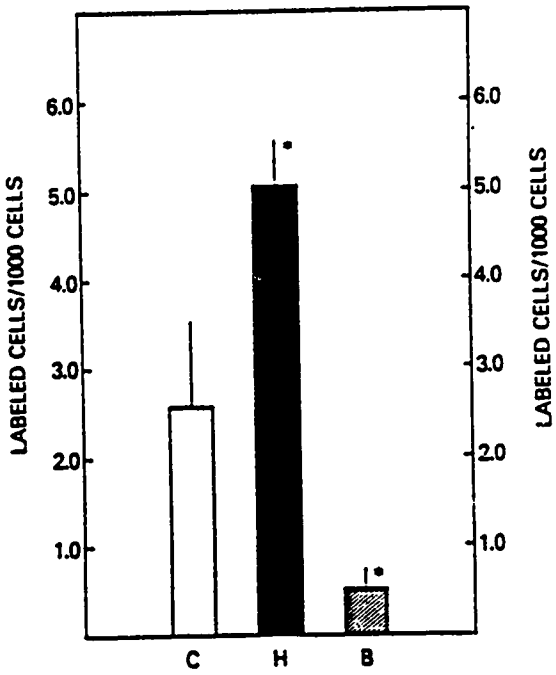
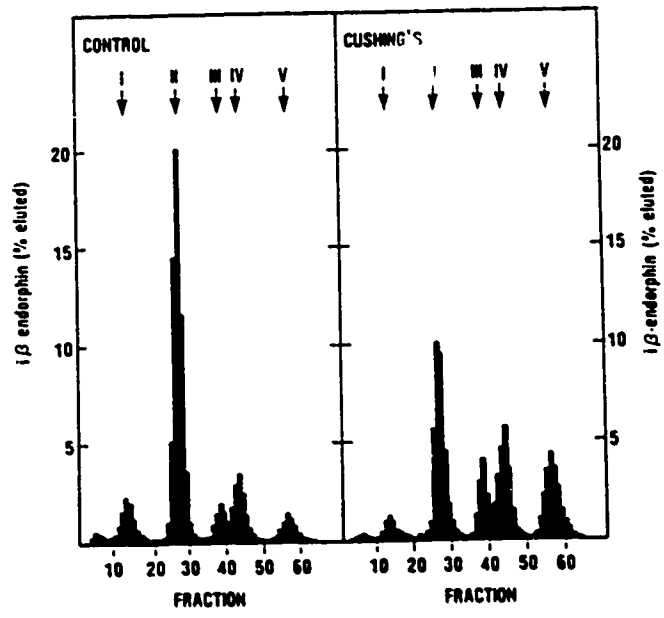


FIGURE 6



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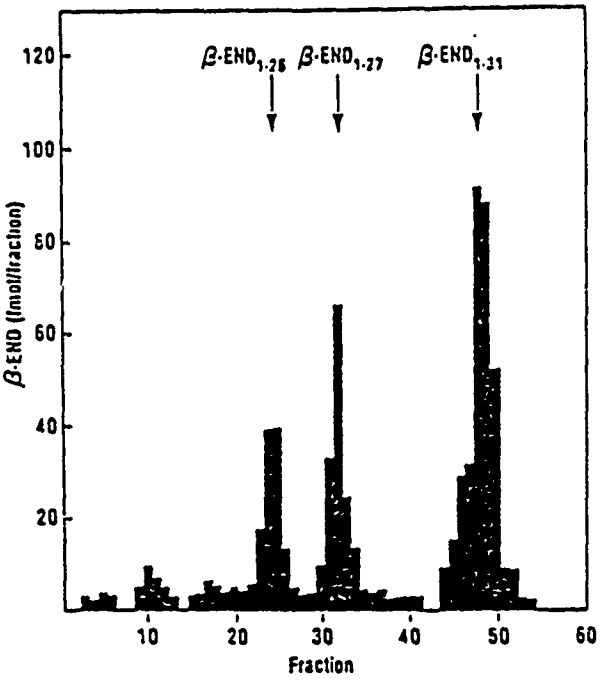

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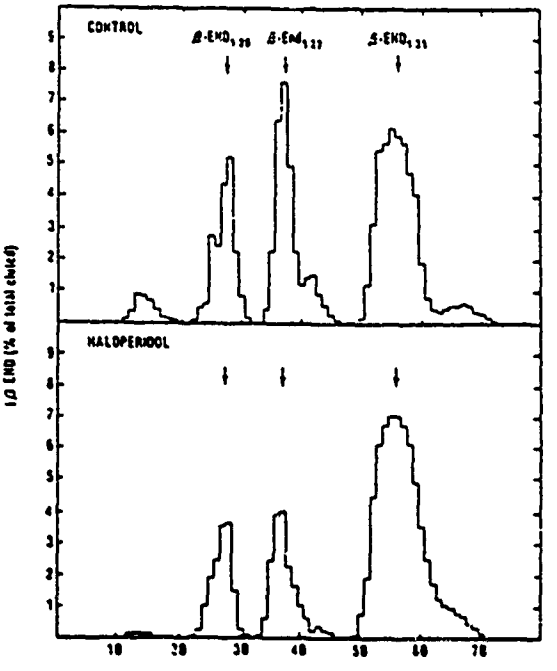

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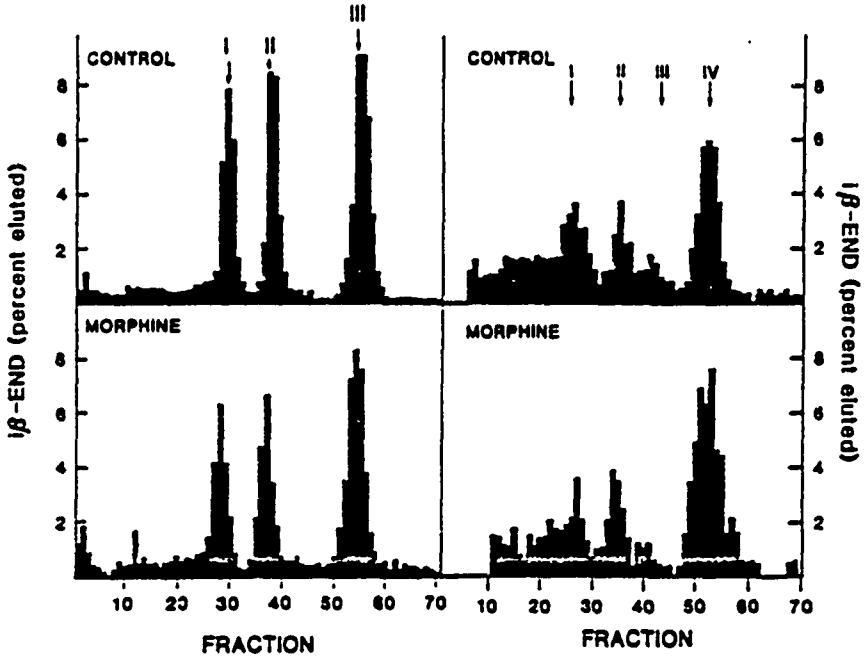


FIGURE 10

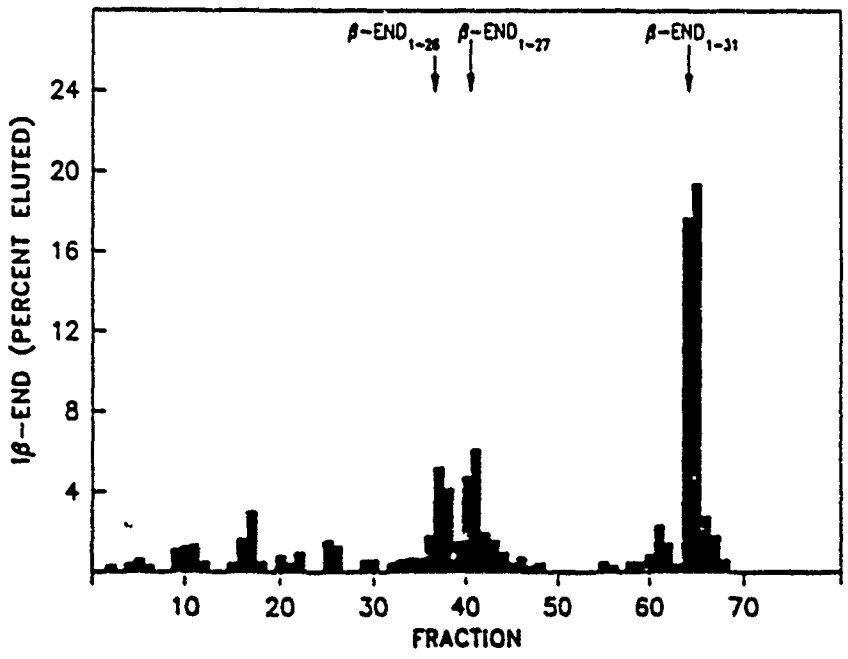
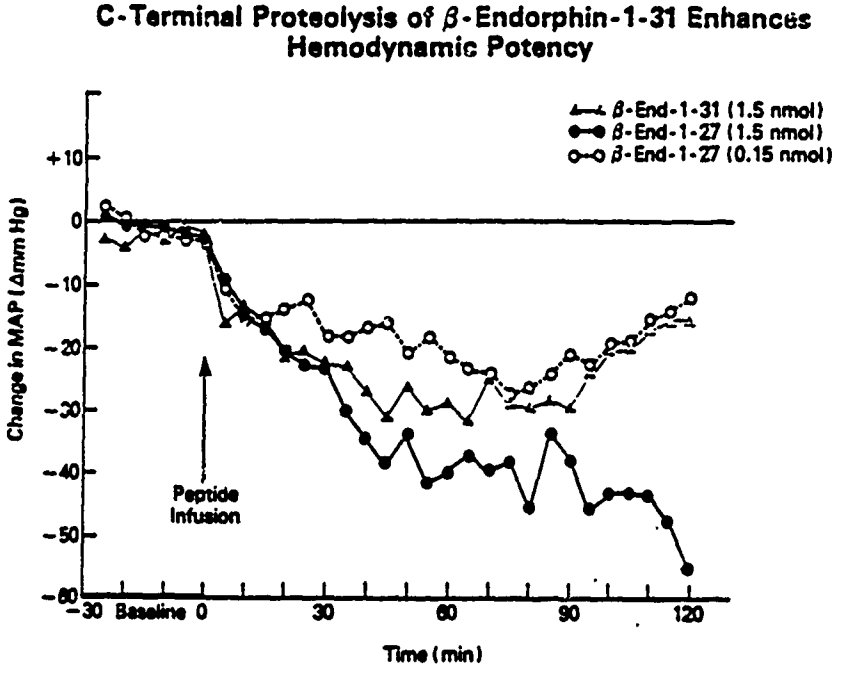


FIGURE 11



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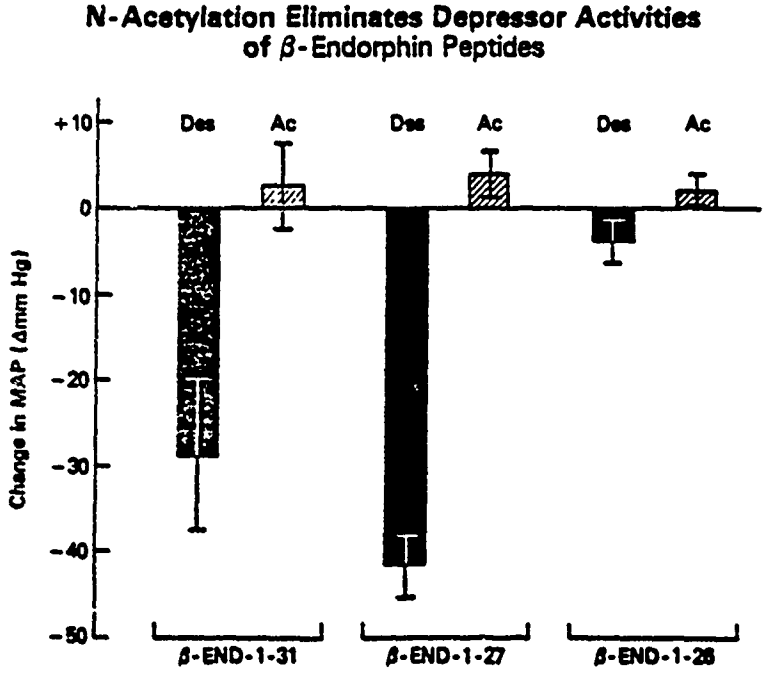

FIGURE 13